A method to type the potential angucycline producers in actinomycetes isolated from marine sponges

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Received: 28 October 2010 / Accepted: 12 January 2011 / Published online: 3 February 2011
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Abstract Angucyclines are aromatic polyketides with antimicrobial, antitumor, antiviral and enzyme inhibition activities. In this study, a new pair of degenerate primers targeting the cyclase genes that are involved in the aromatization of the first and/or second ring of angucycline, were designed and evaluated in a PCR protocol targeting the jadomycin cyclase gene of Streptomyces venezuelae ISP5230. The identity of the target amplicon was confirmed by sequencing. After validation, the primers were used to screen 49 actinomycete isolates from three different marine sponges to identify putative angucycline producers. Seven isolates were positively identified using this method. Sequence analysis of the positive amplicons confirmed their identity as putative angucycline cyclases with sequence highly similar to known angucycline cyclases. Phylogenetic analysis clustered these positives into the angucycline group of cyclases. Furthermore, amplifications of the seven isolates using ketosynthase-specific primers were positive, backing the results using the cyclase primers. Together these results provided strong support for the presence of angucycline biosynthetic genes in these isolates. The specific primer set targeting the cyclase can be used to identify putative angucycline producers among marine actinobacteria, and aid in the discovery of novel angucyclines.

Keywords Actinomycete · Angucycline · Marine sponge · Polyketide synthase

Introduction

As a major source of new natural bioactive compounds, marine sponges harbor large amounts of
bacteria in their tissues that can amount to 40% of their biomass (Vacelet and Donadey 1977; Taylor et al. 2007). And it is widely believed that many of sponges’ products are in fact produced by symbiotic microorganisms (Unson et al. 1994; Kennedy et al. 2007). To exploit the potential bioactive compounds derived from marine sponges, culturing and culture-independent approaches are employed for mining the genetic resources of the sponge-associated microorganisms (Hentschel et al. 2001; Piel et al. 2004; Fieseler et al. 2007), especially those of actinobacteria (Montalvo et al. 2005; Kim and Fuerst 2006; Zhang et al. 2006; Jiang et al. 2007, 2008; Sun et al. 2010).

Actinomycetes are noteworthy for their unmatched secondary metabolite diversity (Jensen et al. 2005). A recent study predicted that the total number of antimicrobial compounds that could potentially be produced by the *Streptomyces* genus is over 100,000, a tiny proportion of which have been unearthed so far (Wawrik et al. 2001). However, the discovery rate of novel bioactive compounds has fallen steadily (Watve et al. 2001), due to a 95% rediscovery rate of known compounds (Fenical et al. 1999). Thus, to speed up discovering microbial natural products, new methods should be developed to evaluate the potential bioactivities of microorganisms.

Angucyclines are a group of aromatic polyketides that have a multitude of properties including antibacterial, antitumor, antiviral and enzyme inhibitory activities (Rohr and Thiericke 1992; Krohn 1997). Some angucyclines show promising activity even against multidrug resistant cancer cells (Korynevskoa et al. 2007). The gene organization in angucycline biosynthesis is similar to that of other aromatic polyketides, with the “minimal PKS” consisting of two ketosynthase (KS) units (KSα and KSβ) and an acyl carrier protein (ACP) as the core, ketoreductase, aromatase and cyclase are the main functional components. The minimal PKS enzymes in collaboration condense small carboxylic acids to form a polyketide chain, then the ketoreductase, aromatase or cyclase converts the elongated polyketide chain into a multicyclic scaffold prior to further tailoring reactions (Schneider 2005). Although protocols employing several sets of primers targeting KS genes have been developed to assess the polyketide biosynthetic gene clusters, which might included angucycline biosynthesis, in actinobacteria (Metsa-Ketela et al. 1999, 2002) and environmental samples (Wawrik et al. 2005, 2007), no method is specifically designed targeting the angucycline biosynthetic genes. In this paper, we designed and evaluated a rapid and specific approach to identify potential angucycline producers based on angucycline-associated cyclases in actinomycetes isolated from marine sponges.

**Materials and methods**

**Bacterial strains and DNA extraction**

*Streptomyces lividans* TK24 and *E. coli* DH5α have been described previously (Chen et al. 2005). Jadamycin-producing *S. venezuelae* has been described by Yang et al. (1995). The type strains of *S. galilaeus* var. siwemensis, *S. nogalater* and *S. albofaciens*, which produce antibiotics aclacinomycin, nogalamycin and oxytetracycline respectively, were obtained from the China General Microbiological Culture Collection Center (CGMCC). Other actinomycete strains used in this experiment were isolated from marine sponges *Haliclona* sp. (Jiang et al. 2007), *Iotrochota* sp. (Jiang et al. 2008) and *Halichondria* sp. The sponge *Halichondria* sp. identified by Chen W. Z. of Shantou University in China was collected at 2 m under the sea water from Shantou, China (23°02′ N, 117°06′ E). Actinobacterial isolates including symbiotic bacteria and those simply adherent to the sponge were obtained using the method described by Zhang et al. (2006). In brief, the bacterial colonies from different culture media bearing typical *Streptomyces*/actinobacterial morphologic characteristics (colorful substrate mycelia, aerial mycelia, spores mass and pigment production) were selected and inoculated onto Gause’s medium No. 1 agar for confirmation of bacterial morphology. The isolates were artificially grouped based on aerial spore mass color, reverse pigment color and the color of any diffusible pigments. The representatives in each group were primarily classified by 16S rRNA gene sequences (Supplementary: Table S1). The total genomic DNA was extracted from all the isolates following the procedures described by Li and De Boer (1995).
Primers targeting the angucycline cyclase gene and PCR optimization

We analyzed the molecular evolution of cyclases involved in the aromatization of polyketides by phylogenetic analysis (Fig. 1). The cyclase phylogenetic tree shows that the separation of different antibiotic groups in distinct branches, particularly the cyclases related to angucycline, were clustered together in a unique subgroup, such as UrdL (GenBank accession number: AAF00205) (Faust et al. 2000), LanL (AAD13540) (Westrich et al. 1999), Sim6 (AAL15584) (Trefzer et al. 2002), aur1H (AAX57195) (Novakova et al. 2002), and jad-cyclase (AAB36566) (Han et al. 1994). The sequences of these gene products were selected as model sequences for designing the primers for angucycline cyclases. The design criteria for the primers were set as follows: (i) no more than 5 nucleotides are degenerated per primer; (ii) last three nucleotides of the 3-end for each primer are not all for G or C; (iii) the length of oligonucleotides for each primer is at least 18 nucleotides.

The two motifs of ENWPRI (UrdL protein position 27–32) and MTLHTG (UrdL protein position 240–245) are conserved enough for designing PCR primers for amplification of angucycline cyclases. The DNA sequences for the degenerate primer pair are AuF3 (5'-GAACTGGCCSARCGTTBTT-3') and AuR4 (5'-CCNGTGTGSARKTCATSA-3'). Initial blasting at GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) found no significant homology of these primer sequences. The primer pairs AuF3 and AuR4, together with IIPF6 (5'-TSGCSTGCTTCGAYGCSATC-3') and IIPR6 (5'-GGANAANCCTCAGCAABBCCGCT-3'), were synthesized by Invitrogen (Shanghai, China). The primer pair IIPF6/IIPR6 was designed to target ketosynthase gene of aromatic polyketide (Metsa-Ketela et al. 1999), PCR conditions were as described by Jiang et al. (2007). The PCR conditions for AuF3/AuR4 were optimized using S. venezuelae ISP5230 as a positive control, and E. coli DH5x, S. galilaeus var. siwenensis, S. nogalater, S. albofaciens and S. lividans TK24 as negative controls. The amplicons of AuF3/AuR4 primers were expected in 600–700 bp size. The annealing temperature of the PCR conditions was finalized at 60°C after testing series of temperatures. The PCR mixture included 10 μl Taq Premix (TaKaRa, Dalian, China), 1 μl each of forward and reverse primers (40 μM each), 5% DMSO (Dimethyl sulfoxide), 0.5–1 μl template DNA, and 6–6.5 μl sterile distilled water in a final volume of 20 μl. Optimized PCR conditions were as follows: (1) denaturation at 94°C for 5 min, (2) 30 amplification cycles with denaturation (45 s, 94°C), annealing (60 s, 60°C), and extension (60 s, 72°C), and (3) a final extension at 72°C for 8 min.

DNA cloning and sequencing

The selected amplicons with primers AuF3/AuR4 (about 600–700 bp in size) or IIPF6/IIPR6 were purified from a 1.0% agarose gel and cloned in E. coli DH5x with pMD18-T Vector (TaKaRa, China) according to the manufacturer’s instructions. After agarose gel verification, five random clones were selected for sequencing using universal primers M13R-28 and M13F-20 by Invitrogen (Shanghai, China). DNA sequences were translated to protein sequences and compared with known sequences in the GenBank database using the BLAST search program (http://www.ncbi.nlm.nih.gov/) (Johnson et al. 2008). The positive threshold value for the obtained sequences when compared to known sequences of cyclases was set at 70%.

Phylogenetic analysis of representative cyclases sequences

Together with the seven putative cyclase sequences amplified in PCR using AuF3/AuR4 primers, 33 representative cyclase sequences retrieved from GenBank were chosen for a phylogenetic and molecular evolutionary analyses using MEGA version 4 (Tamura et al. 2007).

Results

Amplifications of angucycline cyclase and polyketide synthase genes

Under the optimized PCR conditions, a single band about 650 bp size was amplified from S. venezuelae ISP5230 by the primer pair AuF3/AuR4 (Supplementary: Fig. 1S). The amplicon was cloned for sequencing and the results showed that the amplicon of 653 bp in length shares 100% identity with the jad-cyclase gene (AAB36566) involved in biosynthesis
Fig. 1 Neighbor-joining tree of the partial cyclase protein sequences of *Streptomyces* collected from Genbank and the sequences from this work. The tree is comprised of the cyclases forming the first and/or second ring of multicyclic aromatic polyketide. Next to the taxon name, GenBank accession number of cyclase sequence or/and the identified compounds are indicated. The sequences of the branch marked by filled triangles are from this work. The putative ketosynthase gene sequences of the strains in bold were also included in Fig. 2.
of jadomycin B (Han et al. 1994) (data not shown). Using the same conditions, 7 out of 49 actinomycetes isolated from marine sponges were positive in PCR amplification with an amplicon sized around 600–700 bp (Supplementary: Fig. 1S; Table 1S). All the seven amplicons of isolates were cloned for further sequencing analysis. BLAST analysis of the sequencing data showed that all the seven amplicons contain sequences that share sequence similarity of different degrees with known angucycline cyclases (Fig. 1; Supplementary: Table 2S), which also indicates that the amplicons are most likely the putative angucycline cyclases of unknown origin.

Using ketosynthase specific primers IIPF6/IIPR6, we screened 49 actinomycete strains in PCR, and 30 were positive, which including all the seven strains with putative angucycline cyclases amplified using primers AuF3/AuR4. The ketosynthase amplicons of the seven strains were also cloned and sequenced. BLAST analysis of the sequence data showed that all the seven ketosynthase amplicons shared a high similarity with known aromatic KS\textsubscript{a} genes (Fig. 2; Supplementary: Table 2S). Interestingly, some strains, such as \textit{Streptomyces} sp. 15MN1 harbor multiple types of KS\textsubscript{a} genes based on their sequencing data.

Phylogenetic analysis of cyclases sequences with known representatives

Figure 1 illustrates the phylogenetic dendrogram generated from seven putative cyclase sequences obtained in this study, together with 33 representatives of cyclase sequences retrieved from the GenBank. The cyclases shown in the phylogenetic tree (Fig. 1) are mainly involved in the formation of the first and/or second ring in the biosynthesis of multicyclic aromatic polyketide (Han et al. 1994; Ylihonko et al. 1996; Westrich et al. 1999; Faust et al. 2000; Chung et al. 2002), e.g. AknE1 (Chung et al. 2002) and SnoaE (Ylihonko et al. 1996) that are responsible for the aromatization of the first rings in anthracycline biosynthesis. All the cyclases were clustered into subgroups reflecting their aromatic polyketide chemotype (Fig. 1). The Angucycline group, which forms a distinct clade in the tree, contains the cyclase genes involved in the biosynthesis of angucycline antibiotics. For examples urdL (AF164960), lanL (AF080235), sim6 (AAL15584), aur1H AF080235) and jad-cyclase (AAB36566) are involved in the synthesis of the angucycline compounds urdamycin (Faust et al. 2000), landomycin (Westrich et al. 1999), simocyclionone (Trefzer et al. 2002), auricin (Novakova et al. 2002) and jadomycin B (Han et al. 1994), respectively.

The cyclases involved in biosynthesis of isochromanone and anthracyclines form different subgroups in the tree (Fig. 1). Members of each subgroup of the cyclases are associated with the chain length of their cognate polyketides. For example the AknE1 and dpsF were placed in the same subgroup of the tree (Fig. 1), while their corresponding compounds aclacinomycins (Räty et al. 2002) and doxorubicin (Grimm et al. 1994) are similar anthracyclines with the polyketide of the same length of chain (C24). Inconsistencies likely result from horizontal gene transfer, e.g., the cyclase “Med-ORF19” of medermycin (C16) which was probably acquired from another gene cluster (Ridley et al. 2008), is not in the subgroup with the cyclases “gra-orf4” of granaticin (C16) (Bechtold et al. 1995) and “cyclase (CAA45046)” of actinorhodin (C16) (Fernández-Moreno et al. 1992), but in the subgroup of angucycline (Fig. 1). Another exception is that the cyclase “ORF4” of griseusin (isochromanone) is clustered into angucycline group (Fig. 1). The reasons may be ascribed to the facts that griseusin has angular rings framework and its length of the polyketide chain is 20 carbons the same as that of angucyclines such as urdamycin (Faust et al. 2000).

All the seven putative angucycline cyclases from this work are clustered in the angucycline subgroup, indicating that the primers AuF3/AuR4 specifically amplify DNA from angucycline cyclase genes.

Phylogenetic analysis of ketosynthase sequences with known representatives

Using FAB sequence of \textit{E. coli} as an outgroup, a phylogenetic dendrogram (Fig. 2) was generated from 24 reference KS\textsubscript{a} sequences retrieved from GenBank, with eight putative KS\textsubscript{a} sequences obtained in this study and \textit{Jad A} (AAB36562, involved in the biosynthesis of jadomycin B (Han et al. 1994). All the putative KS\textsubscript{a} sequences and \textit{Jad A} are clustered in a group that comprises the angucycline ketosynthases clade with the one exception being \textit{Streptomyces} sp. 15MN1, for which two different ketosynthase fragments were obtained and sequenced. One of them (EU868822) is a putative angucycline ketosynthese, while the other (EU868820) is most likely involved in spore pigment formation (Fig. 2).
Fig. 2  Bootstrap values calculated from 1,000 resamplings using neighbor-joining are shown at the respective nodes when the calculated values were 60% or greater. Neighbor-joining tree of the KS\(\alpha\) gene fragment. Next to the taxon name, GenBank accession number of KS\(\alpha\) sequence or/and the identified compounds are indicated. The sequences of the branch marked by filled triangles are from this work. Bootstrap values calculated from 1,000 resamplings using neighbor-joining are shown at the respective nodes when the calculated values were 50% or greater.

**Discussion**

Aromatases and cyclase often play an important role in biosynthesis of diverse aromatic polyketide scaffoldings (Hopwood 1997). The enzyme, functions like a chaperone, helping direct the nascent polyketide intermediates into particular reaction channels as well as catalyzing C–C bond formations (Hertweck et al. 2007). In some cases, angucycline cyclase demonstrates flexibility for substrates, resulting in the
formation of novel angucyclines in which an ethyl side chain is incorporated into the fourth ring of an anthracycline (Metsa-Ketela et al. 2003). However, because of the difficulties in biochemical investigations of cyclases and their unstable substrates, the specific mechanism for these intriguing cofactor-free enzymes is not clear.

Nevertheless, closely related cyclase proteins often have similar functionality despite the limited knowledge of the precise mechanism of cyclases in aromatic polyketide biosynthesis (Hertweck et al. 2007). For this reason, we analyzed the cyclase sequences from GenBank for their phylogenetic relationship and molecular evolution associated with their chemical identities. The results indicate that the cyclases are divided into different groups, which are characterized by their function and correlative chemotype of aromatic polyketides (Fig. 1). The phylogram of the cyclases in this study (Fig. 1) is largely in agreement with that of KS, chain length factor (CLF), and aromatases reported previously (Metsa-Ketela et al. 2002; Ridley et al. 2008). Especially, angucycline-related KS, CLF, and aromatases form distinct groups (Metsa-Ketela et al. 2002; Ridley et al. 2008), which are strongly supported in this study (Fig. 1 and Fig. 2). Therefore, the angucycline cyclase sequences might be useful to detect angucycline gene clusters and identify potential angucycline producers.

Typing actinobacteria for potential production of a particular type of antibiotics such as angucycline, without going through the tedious chemistry, is more efficient when the typing protocol is targeting the antibiotic biosynthesis gene cluster rather than the taxonomic marker genes (e.g. 16S rRNA gene) which often give misleading results (Metsa-Ketela et al. 2002; Ridley et al. 2008). In this study, degenerate primers, AuF3/AuR4, targeting the cyclase of angucycline was designed and evaluated resulting in a rapid detection of the jadomycin cyclase gene. Further expending the protocol for typing 49 actinobacterial isolates associated with marine sponges revealed seven putative angucycline cyclase genes with sequence confirmation (Fig. 1). The same seven actinobacteria also harbor angucycline KSα gene for aromatic polyketide biosynthesis as a support for the presence of angucycline gene clusters in these bacteria (Fig. 2). Thereby, the protocol for identifying angucycline cyclase using the new primers described in this study can be used to rapidly identify putative angucycline-producing actinobacteria.

To develop new antibiotics for the treatment of antibiotic-resistant pathogens, there has been increasing attention on marine microbial products. Angucyclines are an emerging group of antibiotics that are widely present in actinomycetes (Metsa-Ketela et al. 2002) and environments (Wawrik et al. 2005). However, under laboratory conditions, only few of angucyclines have been recovered from fermentations, mostly with low yield. Thus, most angucycline biosynthetic gene clusters are silent under normal fermentation conditions. Therefore, the metabolites synthesized by angucycline-gene products are difficult to be directly detected. In this paper, we developed a new method to detect the potential angucycline producers, which could facilitate the discovery of new angucyclines by reducing the number of strains that need to be screened by fermentation. (Doull et al. 1993, 1994; Zheng et al. 2007). Further application of the PCR-based screening system will serve as a useful tool for discovering potentially novel angucycline gene clusters in our cosmid and BAC metagenomic DNA libraries (data not shown) generated from deep marine environmental samples. Future work will concentrate on chemical identification of the angucyclines produced by the strains identified in this study.

Acknowledgments This research was partially funded by the State Principal and Basic Research and Development Program of the Ministry of Sciences and Technology of China (2010CB833801-3) and Provincial Collaborative Foundation Project of Guangdong (9351007002000001, 2008A030203004). We also thank the financial support of the China Ocean Mineral Resources R&D Association Project (DYXM115-02) and the Open Project Program (LMB091001) of the Key Laboratory of Marine Bio-resources Sustainable Utilization, SCSIO-CAS. Many thanks are given to Dr Ravi Kumar for critical reading of this manuscript and Chen W. Z. for identifying the marine sponge.

References
Expression, purification, and characterization of AknX anthrone oxygenase, which is involved in aklavinone biosynthesis in *Streptomyces galilaeus*. J Bacteriol 184:6115–6122


